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An iron binding assay to measure activity of known food sequestering agents: studies with buttermilk solids

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Abstract

A method enabling the measurement and differentiation of both ferrous (Fe^{2+}) and ferric (Fe^{3+}) binding was developed to assess the relative sequestering power of known iron chelators and buttermilk solids (BMS). Fe^{2+} and Fe^{3+} chelation by BMS (0.1–10.0 mg), phytic acid, ethylenediaminetetraacetic acid (EDTA) and citric acid (0.1 mM) was measured using a modification of a ferric-thiocyanate method. Fe^{2+} and Fe^{3+} was determined from the amount of unbound ferric-thiocyanate following incubation with chelator. The assay was optimized for polarity of solvent and pH. Fe^{2+} and Fe^{3+} binding by sequestering agents was, in descending order of affinity to be: phytic acid > EDTA > BMS > citric acid. Known food iron chelators had a higher affinity towards Fe^{3+}, while BMS had a higher affinity towards Fe^{2+}. BMS also demonstrated an excellent iron solubilization property through iron binding activity. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Ferrous; Ferric; Iron binding; Phytic acid; EDTA; BMS

1. Introduction

Ferrous (Fe^{2+}) ion is recognized as the biologically active form of iron due to its potent oxidizing activity. In the presence of lipids, Fe^{2+} induces lipid oxidation leading to off-flavour and off-odour in foods. Furthermore, Fe^{2+} can react with hydrogen peroxide to produce hydroxyl radical in the Fenton reaction (Wardman & Candeias, 1996). In comparison, the ferric (Fe^{3+}) ion is the relatively biologically inactive form of iron; however, Fe^{3+} can be reduced to the active Fe^{2+} form by reducing agents, such as ascorbic acid or certain plant phenolics, which in turn catalyzes degradative reactions. One example is the Haber–Weiss Cycle, where the Fe^{3+} is reduced to Fe^{2+} by superoxide anion (Wardman & Candeias). The reduction of Fe^{3+} to Fe^{2+} ion, thus enables participation in the Fenton reaction and the production of hydroxyl radicals.

The prevention of iron-induced oxidation reactions can be accomplished in part by the chelation or sequestering of free ion. Whey proteins, comprising β-lactoglobulin and lactoferrin, have been shown to exhibit antioxidant activity due to iron chelation by lactoferrin and copper chelation by β-lactoglobulin (Donnelly, Decker & McClements, 1998). Other researchers have shown that added iron can be bound to casein and distributed within the micelle (Hekmat & McMahon, 1998). In particular, Demott and Dincer (1976) determined that 72% of added iron was bound to α_s-casein. However, there has been little research describing the iron binding affinity of buttermilk solid, a crude and waste product from butter making.

Many researchers have cited various techniques employed to study iron binding activity by specific chelating agents used in the food industry. These methods include iron binding involving the use of radioactive isotope of iron, particularly $^{59}FeCl_3$ (Basch, Jones, Kalan & Wondolowski, 1974; Demott & Dincer, 1976; King, Luick, Litman, Jennings & Dunkley, 1959; Vaughan & Knauff, 1961). This technique requires the use of radioisotopes and thus the form of iron distributed in a complex molecule is often not reported. Alternatively, Hekmat and McMahon (1998) used electron microscopy to determine the distribution of added iron in skim milk and yogurt. The absorption spectrum of an iron–chelator complex has also been studied (Charley,

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Sarkar, Stitt & Saltman, 1963; Zhang et al., 1996), where a change in the absorbance over a range of wavelength absorbance maxima specific to the iron–chelator complex is used to estimate iron binding. Chemical assays involving colour development of free iron after the addition of chelator, or following precipitation, has also been documented (Kearsley & Birch, 1977; Smith and Miller, 1998). Colour development usually has a broad wavelength range and the precipitation from an iron–chelator complex may not always be present if the iron–chelator complex is soluble. The objective of this study was to develop a simple and reproducible method to measure and differentiate between Fe^{2+} and Fe^{3+} binding activity of common food sequestering agents. Specifically, we were also interested in comparing the iron sequestering activity of BMS using this method. We report the relative iron binding potential of buttermilk solids to various synthetic and natural metal chelators using this method.

2. Materials and methods

2.1. Materials

Spray-dried buttermilk solid (BMS) was donated by Dairyworld Foods (Burnaby, BC) and stored in a polyethylene bag at 4°C until used. Ferrous chloride, ferric chloride and L-ascorbic acid were obtained from BDH Chemicals Co. (Toronto, ON). Ethanol, hydrogen peroxide and ammonium thiocyanate were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Na_2-EDTA, citric acid and phytic acid were purchased from Sigma Chem. Co (St. Louis, MO). All water used was distilled deionized water treated with Chelax-100.

2.2. Iron binding assay

The rapid colorimetric assay used for measuring iron binding in this study is a modification of the ferric-thiocyanate method of Duh and Yen (1995). This procedure was originally designed for measuring lipid peroxide content in an emulsion system, whereby the end-point measure is the amount of Fe^{2+} that is oxidized to Fe^{3+} by lipid peroxides. The Fe^{3+}-thiocyanates complex produces a deep red colour, which is detectable at 500 nm. The advantage of using ammonium thiocyanate over other coloring reagents, such as tannic acid, is that binding of iron by thiocyanate ion is specific to the Fe^{3+} only, and that the Fe^{3+}–thiocyanate complex produces a single absorbance peak at 500 nm. Other known coloring reagents such as bathophenanthroline and ferrozine have a binding affinity towards Fe^{2+} only, but can be adapted through our procedure to differentiate between Fe^{2+} and Fe^{3+} during chelation studies. However, meticulous measures must be taken to prevent oxidation of Fe^{2+} to Fe^{3+} by light and oxygen while conducting the experiment that can lead to an overestimation of iron binding potential of chelating agents.

Our modification of this procedure included adding 0.1 ml aliquot of $FeCl_2$ or $FeCl_3$ solution (1.0, 2.5 and 5.0 mM in 3.5% HCl) and 0.1 ml of test samples (e.g. BMS (0.1–10 mg), EDTA (0.1 mM), citric acid (0.1 mM) and phytic acid (0.1 mM); dissolved in 1 mL distilled deionized water) to 5 mL of 75% ethanol. Mixtures were vigorously vortexed and allowed to stand for 1 h at room temperature in the dark. After incubation, 0.1 mL of 30% H_2O_2 was added to the mixture and the reaction incubated for another 5 min. To measure free Fe^{3+} in solution, 0.1 mL ammonium thiocyanate (30%) was added and the reaction kept at room temperature for 5 min before reading absorbance at 500 nm. The difference between the known amount of iron added to the reaction and the total amount of free iron measured after incubation represented the proportion of insoluble Fe^{3+}.

The addition of H_2O_2 to the sample was employed to convert reduced Fe^{2+} to Fe^{3+}, enabling differentiation between Fe^{2+} and Fe^{3+} in the assay. To measure the amount of Fe^{2+} in solution after incubation, duplicate samples were used. One sample was analyzed for Fe^{3+} content in solution after incubation without the addition of H_2O_2. The second sample followed the exact procedure mentioned above to determine Fe^{3+} after oxidation by H_2O_2. The differences between the two samples represented the amount of soluble Fe^{2+} in solution.

2.3. Effects of incubation time

Since iron binding by known chelators may be affected by time, the effect of incubation time on binding 5.0 mM Fe^{3+} was determined. In this experiment, we used samples which were periodically removed at 10 min intervals, over 60 min for analysis using the procedure outlined above. Results are compared to the known amount of iron added to the control tube.

2.4. Effects of solvent

Buffers such as phosphate buffer have been shown to chelate iron and interfere with iron binding assay (Graft, Mahony, Bryanti & Eaton, 1984). Therefore, several solvents were tested for possible effect on Fe^{3+} binding using 5.0mM $FeCl_3$. Distilled deionized water, 0.2M Tris buffer at pH 4.5 and 0.1M HEPES buffer at pH 4.5 were used with no further modifications to the procedure.

2.5. Effects of pH

Since the solubility of iron is pH sensitive, various buffer pHs were examined to determine the optimum pH for iron binding. Both 0.2M Tris and 0.1M HEPES

buffers were adjusted to pH values of 4.6, 5.0, 5.5, 6.0, 6.5 and 7.0, respectively. Ethanol (75%) and distilled deionized water had pH values of 5.6 and 5.5, respectively.

2.6. Effects of polarity

The effect of polarity (i.e. the concentration of ethanol) was also examined. Ethanol was diluted to concentrations of 0, 15, 30, 45, 60 and 75% for analysis of Fe^{3+} binding by buttermilk solid.

2.7. Standard curve

Standard curves for the absorbance of Fe^{2+} and Fe^{3+} were determined using a 0.1–60 μg concentration range of $FeCl_2$ and $FeCl_3$, respectively. The equation relating the concentrations of iron to the absorbance values at 500 nm for Fe^{2+} was $y = 0.0262x + 0.0165$ ($R^2 = 0.999$) and for Fe^{3+} was $y = 0.0418x - 0.0085$ ($R^2 = 0.998$).

2.8. Statistical analysis

All treatments were done in triplicate and each experiment was conducted twice. Data were analyzed by one-way ANOVA and Tukey's test for significance at a probability of $P < 0.05$, using the MiniTab statistical program (MiniTab Inc., PA).

3. Results and discussion

3.1. Absorption spectra

The absorption spectrum of mixing Fe^{3+} with ammonium thiocyanate is shown in Fig. 1. The deep red colour of the Fe^{3+}–thiocyanate complex is detected between 400 to 600 nm, with a maximum absorbance at 500 nm (Duh & Yen, 1995). With the addition of BMS, containing a variety of different molecular weight substances, the maximum absorbance of the Fe^{3+}-thiocyanate was also found to be at 500 nm. This result indicated that possible interference by minute amounts of iron present in the BMS, or other unknown interactions of the constituent of BMS with the thiocyanate ion were minimal. The addition of known food-grade iron chelators also had no effect on the detection of Fe^{3+}–thiocyanate at 500 nm.

3.2. Effects of time

According to Hekmat and McMahon (1998), the binding of Fe^{3+} to casein over a pH range of 5.6–7.8 occurs instantaneously and is thermodynamically favourable due to a small and negative change in free energy. However, Smith and Miller (1998) reported that the binding of Fe^{3+} by tannic acid (a natural iron chelator from tea) increased over time. This finding suggests that an optimal time for Fe^{3+} binding to a specific chelator should be determined. The Fe^{3+} binding potential for BMS over a 60 min period is shown in Fig. 2. Maximum levels of Fe^{3+} binding occurred after 10–20 min of incubation for all levels of BMS tested, suggesting that the range of BMS tested (between 0.1 and 10.0 mg) had no effect on the time required for maximum binding. Increasing the incubation time also did not increase the amount of bound Fe^{3+}.

3.3. Effects of solvent selection

Sodium and potassium phosphate buffers have been shown to interfere with Fe^{3+} binding by the formation of insoluble complexes with dissolved iron (Kamasaka, To, Kusaka, Kuriki, Kometani & Okada 1997). In this study, a comparison between 75% EtOH, deionized distilled water, Tris and HEPES buffers on available Fe^{3+} (27.93 μg) in solution is shown in Fig. 3. No significant difference between the detected amount of recovered bound Fe^{3+} in 75% EtOH and the actual quantity originally added was found. Substituting EtOH with water or buffer significantly ($P < 0.05$) reduced the measured estimate of bound Fe^{3+} to approximately half of the actual quantity present. The relative order of Fe^{3+}–thiocyanate formed complexes in these solvents was, water > Tris > HEPES. Since both ammonium thiocyanate and $FeCl_3$ are soluble in ethanol and water (Merck Index, 1968), the recovery of Fe^{3+} after incubation should theoretically be equal to that of 75% EtOH. However, reactivity of solubilized iron is lower in our neutral pH solutions tested such as water, Tris and HEPES buffer due to formation of iron hydroxides (Smith, 1983) and thus become less reactive with the thiocyanate ion. Ethanol at 75% concentration was found to have a pH value of 5.8 and therefore prevented the formation of less reactive iron hydroxides. Therefore, an assessment of the effects of pH on iron solubility under our test condition was conducted to determine optimum pH conditions to study iron chelation.

3.4. Effects of pH

Since the solubility of iron is markedly influenced by pH (Smith, 1983), it is possible that the formation of the Fe^{3+}–thiocyanate complex could also be affected by pH. The effect of varying buffer pH at 4.6–7.0 on the determination of the Fe^{3+}–thiocyanate complex is shown in Fig. 4. Varying pH values from 4.6 to 5.5, had no effect on the measurement of the Fe^{3+}–thiocyanate complex. This finding is supported by the fact that iron in solutions of low pH are highly soluble as hydrates of $Fe(H_2O)_6^{2+}$ and $Fe(H_2O)_6^{3+}$ (Clydesdale, 1983). In contrast, $FeCl_3$ solubility is very low at neutral pH due to the formation of hydroxides [e.g. $Fe(OH)_3$] that result

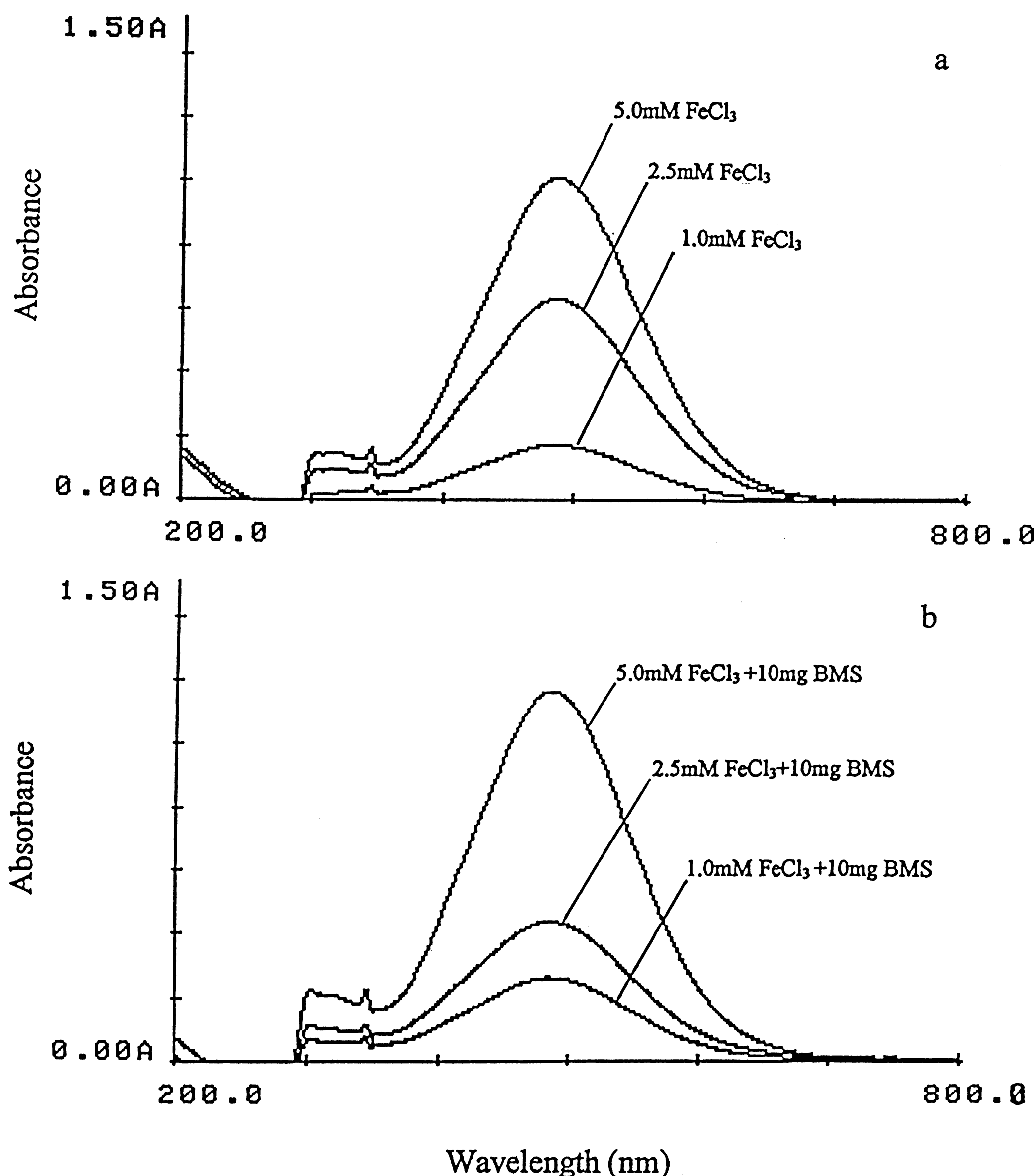

Fig. 1. (a) Absorbance spectra for ferric thiocyanate complex; (b) absorbance spectra for ferric thiocyanate complex with buttermilk solids (BMS).

from oxidation (Clydesdale). This fact was observed when the measured quantity of the Fe^{3+}–thiocyanate complex began to decrease at pH values of 6–7. At neutral pH, only 1.2 μg of Fe^{3+} was recovered from the original amount (27.93 μg) added. Both Tris and HEPES buffers also exhibited similar recoveries of Fe^{3+} over the pH ranges tested. It was concluded that a pH range of 4.6–5.5 was optimal for determining the Fe^{3+} –thiocyanate complex.

3.5. *Effects of polarity*

Of all the solvents examined, ethanol had the highest polarity, with water, Tris and HEPES buffers all being relatively less polar than ethanol. The possibility that the polarity of the solvent might be a critical factor when choosing the right medium for analysis was evident when all three polar solvents failed to reproduce the recovery of added Fe^{3+}. A range of concentrations of ethanol (e.g. 0–75%), thus reflecting the polarity of the solvent used, was evaluated for determining the effect on Fe^{3+} binding by sequestering agent on all three concentrations of $FeCl_3$ (Fig. 5). The amount of Fe^{3+} recovered was similar for all three concentrations of iron tested in solvents with measured proportions of ethanol. For example, a range of ethanol from 0–60% produced results that were significantly different ($P < 0.05$) from solvents containing 75% ethanol. Optimal recovery of Fe^{3+} was obtained at 75% ethanol. The effect of polarity of ethanol on Fe^{3+} determination is attributed to the acidity of the molecule, since ethanol is essentially a weak acid due to its ability to deprotonate to form ethoxide. A higher concentration of

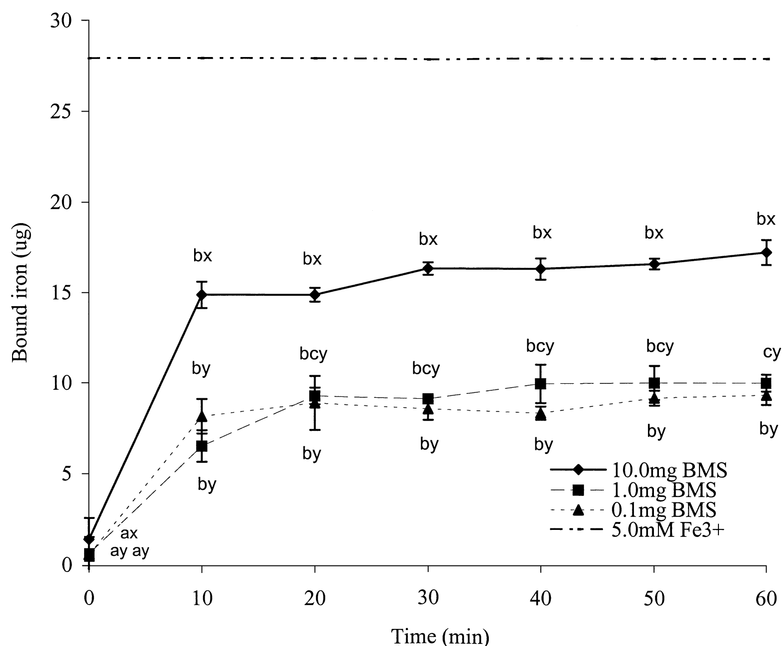

Fig. 2. Effects of incubation time on ferric iron binding by buttermilk solids (BMS). All values represents mean±SEM. [abc]Data within a treatment with different letter are significantly different ($P<0.05$). [xy]Data between treatments with different letter are significantly different ($P<0.05$). — ◆ —: 10 mg BMS; — ■ — 1.0 mg BMS; ···▲··· 0.1 mg BMS; — ·· — ·· — 5.0 mM Fe^{3+}.

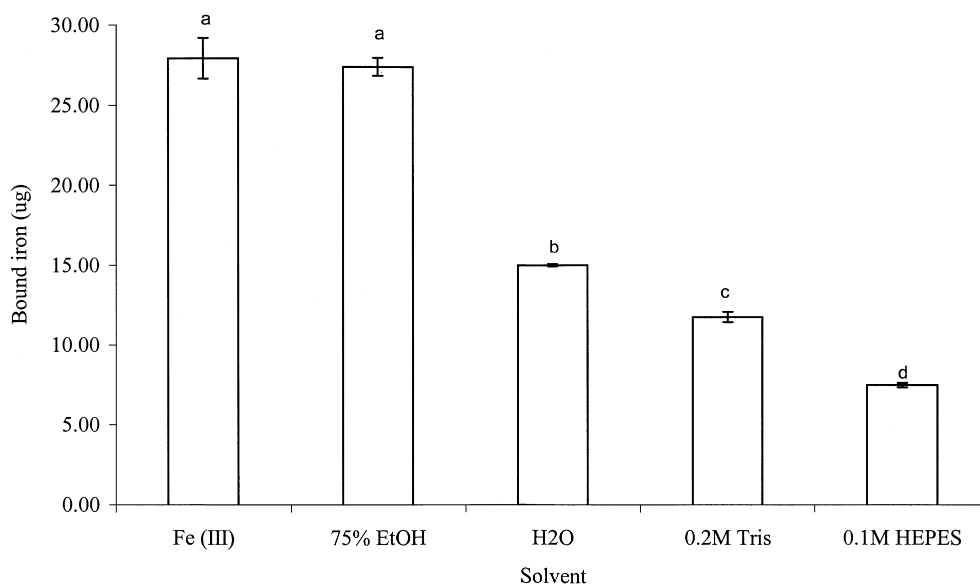

Fig. 3. Effects of solvent on ferric iron binding by buttermilk solids (BMS). All values represents mean±SEM. [abcd]Data within a treatment with different letter are significantly different ($P<0.05$).

ethanol would therefore deprotonate to a greater extent, thus resulting in increased Fe^{3+} solubility under the acidic conditions. Given these results, it was determined that the solvent of choice for our assay should have a polarity equal to or greater than 75% EtOH.

3.6. Iron binding by synthetic and natural chelators

Fe^{2+} and Fe^{3+} chelation or sequestering by 0.1 mM of phytic acid, EDTA and citric acid is shown in Figs. 6 and 7, respectively. In our study, 0.1 mM of phytic acid was found to bind between 85 and 95% for both added Fe^{2+} and Fe^{3+} at a phytic acid to iron ratio equivalent or greater than 1:25. An earlier study by Graft and Eaton (1990) also reported a high affinity of Fe^{3+} by phytic acid and that low concentrations of phytic acid in comparison to Fe^{3+} generally form insoluble $Fe(III)_3$– and $Fe(III)_4$–phytate complexes. Further additions of phytic acid increased the phytate ratio to iron ratio to 1:50 produced a drastic decline in iron binding to 43% with

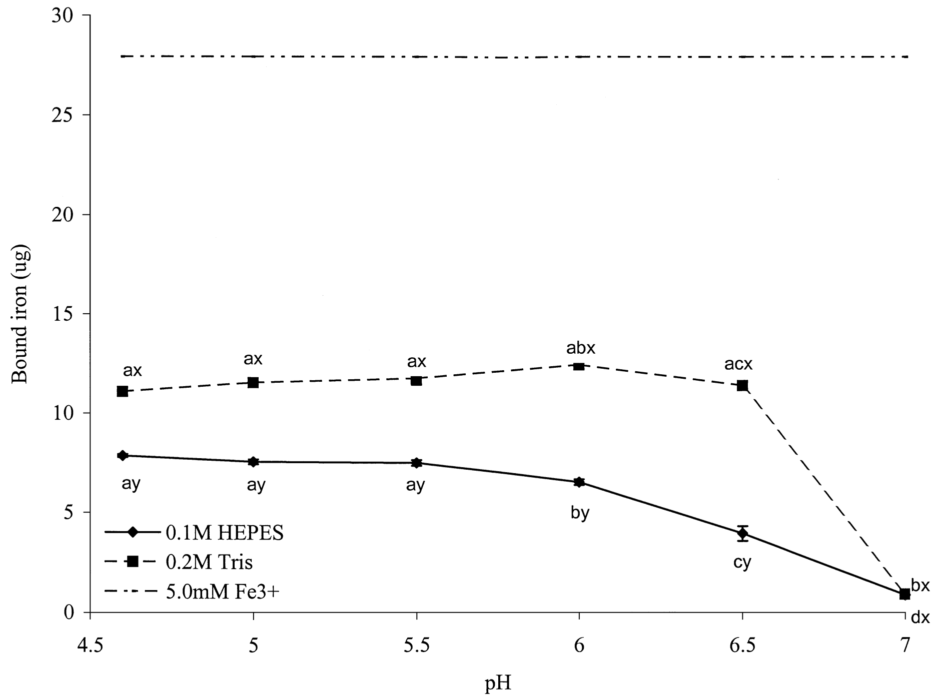

Fig. 4. Effects of buffer pH on ferric iron binding by buttermilk solids (BMS). All values represents mean + SEM. [abcd]Data within a treatment with different letter are significantly different ($P < 0.05$). [xy]Data between treatments with different letter are significantly different ($P < 0.05$). — ◆ — 0.1M HEPES; — ■ — 0.2M Tris; — ·· — ·· — 5.0 mM Fe^{3+}.

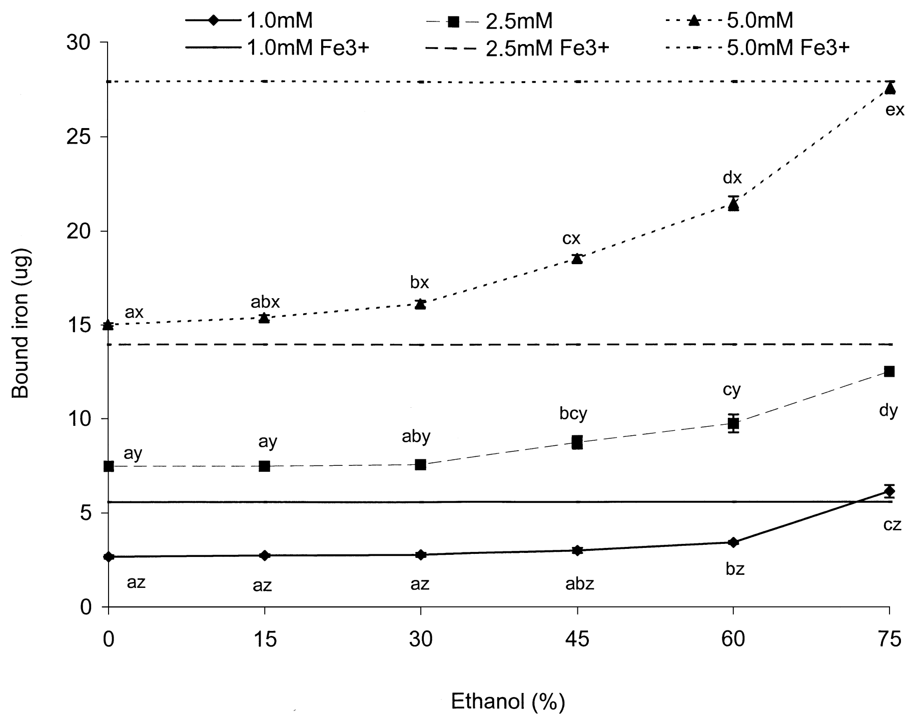

Fig. 5. Effects of ethanol polarity on ferric iron binding by buttermilk solids (BMS). All values represents mean±SEM. [abcde]Data within a treatment with different letter are significantly different ($P < 0.05$). [xyz]Data between treatments with different letter are significantly different ($P < 0.05$). Lines with symbol represent recovery of added iron at various concentrations of ethanol and lines without symbol represents initial amount of added iron. — ◆ — 1.0 mM; — ■ — 2.5 mM; ···▲··· 5.0 mM; — — - 1.0 mM Fe^{3+}; - - - - - - 2.5 mM Fe^{3+}; — ·· — ·· — 5.0 mM Fe^{3+}.

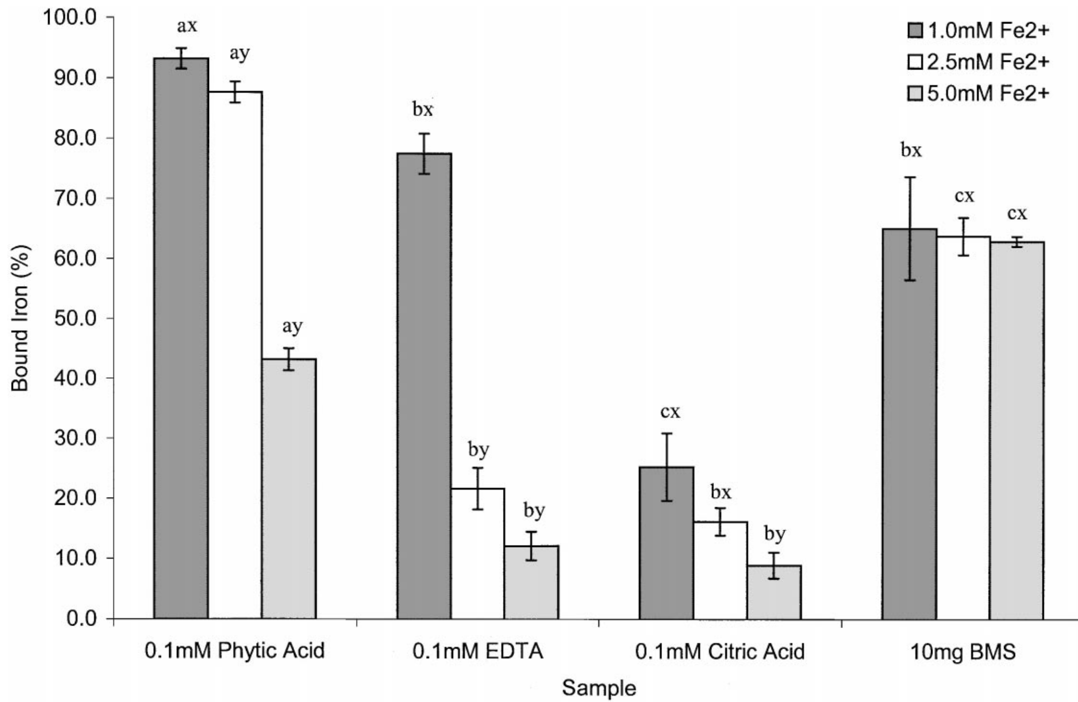

Fig. 6. Comparison of ferrous iron binding between chelator and buttermilk solids (BMS). All values represents mean±SEM. [abc]Data within a treatment with different letter are significantly different ($P<0.05$). [xy]Data between treatments with different letter are significantly different ($P<0.05$). ■ 1.0 mM Fe^{2+}; □ 2.5 mM Fe^{2+}; ■ 5.0 mM Fe^{2+}.

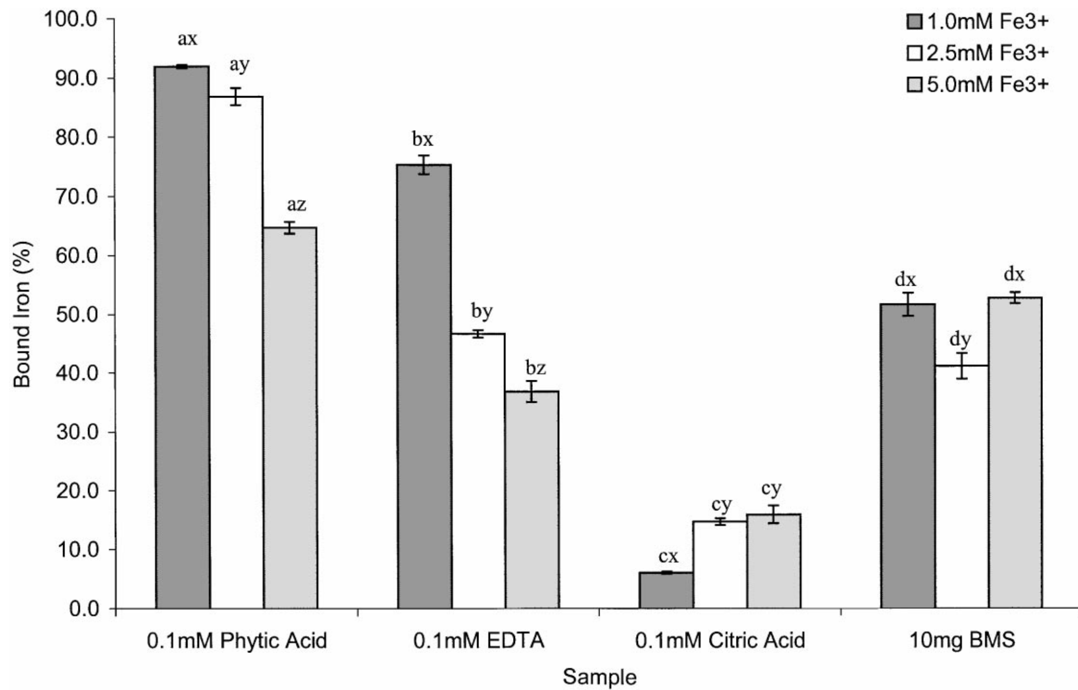

Fig. 7. Comparison of ferric iron binding of food chelator and buttermilk solids (BMS). All values represents mean±SEM. [abcd]Data within a treatment with different letter are significantly different ($P<0.05$). [xyz]Data between treatments with different letter are significantly different ($P<0.05$). ■ 1.0 mM Fe^{2+}; □ 2.5 mM Fe^{2+}; ■ 5.0 mM Fe^{2+}.

Fe^{2+} and 65% with Fe^{3+}. This marked decline in binding affinity for Fe^{2+} by phytate may be the result an accelerated oxidation of Fe^{2+} to Fe^{3+} mediated by phytate occurred at this concentration (Graft, Empson & Eaton, 1987). It is noteworthy that a high phytate to iron ratio can impart a dual antioxidant function, due to both sequestering iron and increasing the depletion of Fe^{2+} through shifting the redox potential of iron. In either case, the phytic acid binding affinity for Fe^{2+} was significantly greater than that of BMS.

EDTA demonstrated a relatively lower affinity for both Fe^{2+} and Fe^{3+} than phytic acid (Figs. 6 and 7). An EDTA to iron ratio of 1:10 exhibited the highest iron binding affinity at approximately 78%. Hsieh and Hsieh (1997) observed that the optimum formation of an EDTA–Fe^{3+} complex occurred at a ratio of 1:1. Interestingly, the affinity of binding for 2.5 and 5.0 mM of Fe^{3+} by EDTA was shown to be significantly higher than for Fe^{2+}. Since the spontaneous oxidation of Fe^{2+} in solution near neutral pH is relatively slow (Graft & Eaton, 1990), it is expected that large concentrations of Fe^{2+} were not as efficiently chelated by EDTA. Furthermore, maximum chelating potential would be expected at a higher pH value, where the EDTA carboxyl groups are fully dissociated (Dziezak, 1986). The relatively low pH (i.e. 5.5) of the reaction medium might also be responsible for a low binding affinity observed for both Fe^{2+} and Fe^{3+} to EDTA, since at this pH, the non-dissociation of the four carboxylic groups in EDTA occurs.

Among the three well-known sequestering agents, citric acid had the lowest affinity for iron (Figs. 6 and 7). In comparison to EDTA, citric acid has been reported to be less effective than EDTA in chelating metals (Gordon, 1990). However, citrate still remains a highly effective sequestrant in many food systems (Dziezak) and is twice as effective in decreasing Fe^{3+} than Cu^{2+} mediated lipid oxidation (Gordon). Similar to EDTA, citric acid sequestering activity of metal ion occurs with available carboxyl groups as evidenced by the reduced sequestering power due to increased esterification of the carboxyl groups (Gordon).

3.7. Iron binding by BMS

The affinity of BMS to chelate Fe^{2+} is shown in Fig. 8. In general, Fe^{2+} chelation by BMS was high, ranging between 55 and 95% recovery of the originally added iron. All concentrations of BMS exhibited a similar affinity to bind Fe^{2+}. A comparison of mean by Tukey's test revealed that BMS concentrations between 1.0 and 7.5 mg tended to bind 1.0 mM of Fe^{2+} significantly ($P<0.05$) more than either 2.5 or 5.0 mM, and that binding to 2.5 and 5.0 mM by the higher concentrations of BMS (>1.0 mg) tested was on the whole statistically not different ($P>0.05$). This suggested that iron binding by BMS approached a maximum when the iron level is increased to between 2.5 and 5.0 mM Fe^{2+}. There was no correlation found between the percentage of iron bound and the concentration of BMS, thus indicating

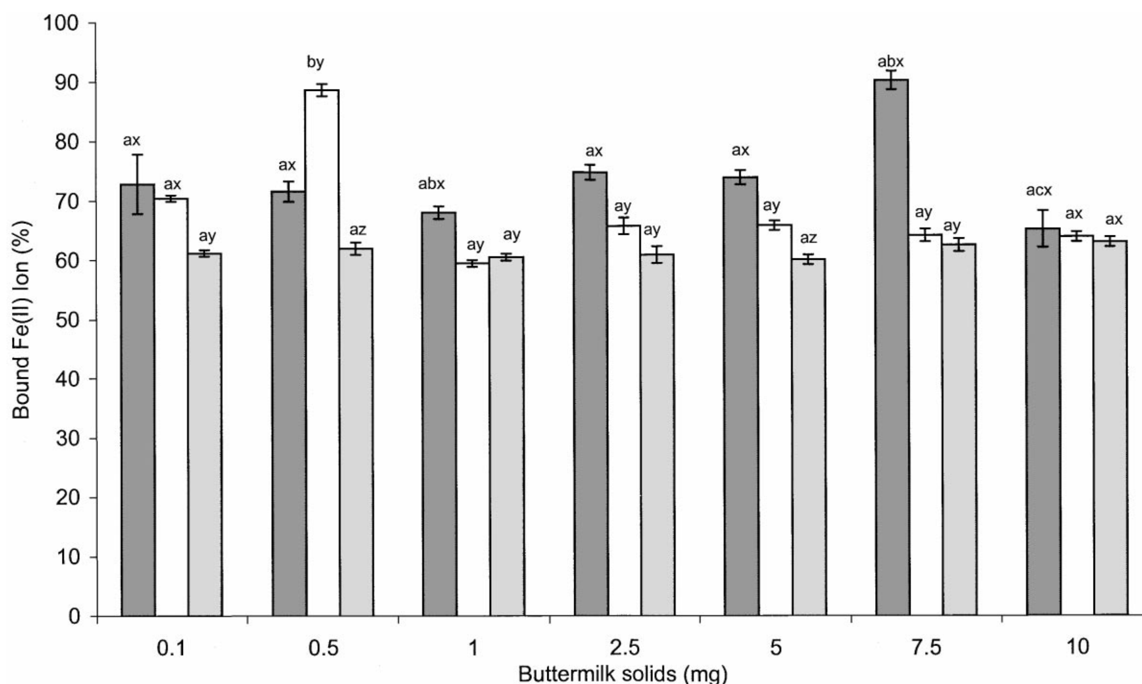

Fig. 8. Ferrous iron binding by buttermilk solids. All values represents mean±SEM. [abc]Data within a treatment with different letter are significantly different ($P<0.05$). [xyz]Data between treatments with different letter are significantly different ($P<0.05$). ■ 1.0 mM Fe^{2+}; □ 2.5 mM Fe^{2+}; ▢ 5.0 mM Fe^{2+}.

that Fe^{2+} binding saturation had been reached at a relatively low concentration of BMS.

The affinity of BMS to bind Fe^{3+} is shown in Fig. 9. In general, BMS bound less Fe^{3+} (18–55%) than Fe^{2+} (55–95%). Over a 0.1 to 2.5 mg range of BMS, the percentage of bound iron was not significantly different ($P>0.05$) for all concentrations of added $FeCl_3$. Increasing the concentration of BMS from 5.0 to 10.0 mg, however, did increase ($P<0.05$) the percentage of bound iron, thereby suggesting a different affinity for Fe^{3+} recovery at different levels of BMS.

Several researchers have investigated the ability of casein and whey to binding Fe^{3+} (Basch et al., 1974; Demott and Dincer, 1976; Demott and Park, 1974; Hekmat and McMahon, 1998; McMahon and Brown, 1984; Vaughan and Knauff, 1961). Demott and Park reported that 87% of added Fe^{3+} was bound by raw and pasteurized skim milk. Isoelectric casein and whey bound an estimated amount of 67.84 and 4.54%, respectively, of the total bound iron. These former studies confirm the potential for BMS in forming soluble chelates of both Fe^{2+} and Fe^{3+} sources.

Ferrous ion is the reduced and biologically active form of iron, which is central to the induction of lipid and protein oxidation by the formation of •OH via the Fenton reaction. Therefore, the affinity of BMS to bind Fe^{2+} is critical for this material to have an application to inhibit oxidative damages in food. On the other hand, Fe^{3+} is the oxidized and biologically inactive form of iron and the presence of Fe^{3+} in foods in general has a lesser role in the induction of oxidative degradative reactions. This fact could lead to the suggestion that chelation of Fe^{3+} may therefore have little relevance in affecting lipid oxidation. This in fact is not the case, if free Fe^{3+} was in contact with a reducing agent, which would result in Fe^{3+} being reduced back to the active Fe^{2+} form and thereby leading to the potential participation in free radical production and lipid oxidation reactions. Two such examples of redox-Fe dependent lipid oxidation can be found in meat and dairy products. Lee, Hendricks and Cornforth (1999) observed an increase in lipid oxidation as a result of adding 0.05% ascorbic acid to ground beef patties and have attributed the cause of this increase in oxidation to the reduction of the inactive Fe^{3+} to the active Fe^{2+} by ascorbic acid as proposed by Miller and Aust (1989). Hegenauer, Saltman, Ludwig, Ripley and Bajo (1979) also reported an increase in lipid oxidation of milk supplemented with copper and iron and suggested that the potential of Fe^{2+} to catalyze lipid oxidation may be amplified through the cyclic redox reactions with reducing agents present in milk. Our assay enables this potential dual activity of sequestering agents to be evaluated for this possible activity. The ability of BMS to chelate Fe^{3+}, and furthermore inhibit Fe^{2+} transformation is therefore an important role in controlling oxidative changes in food.

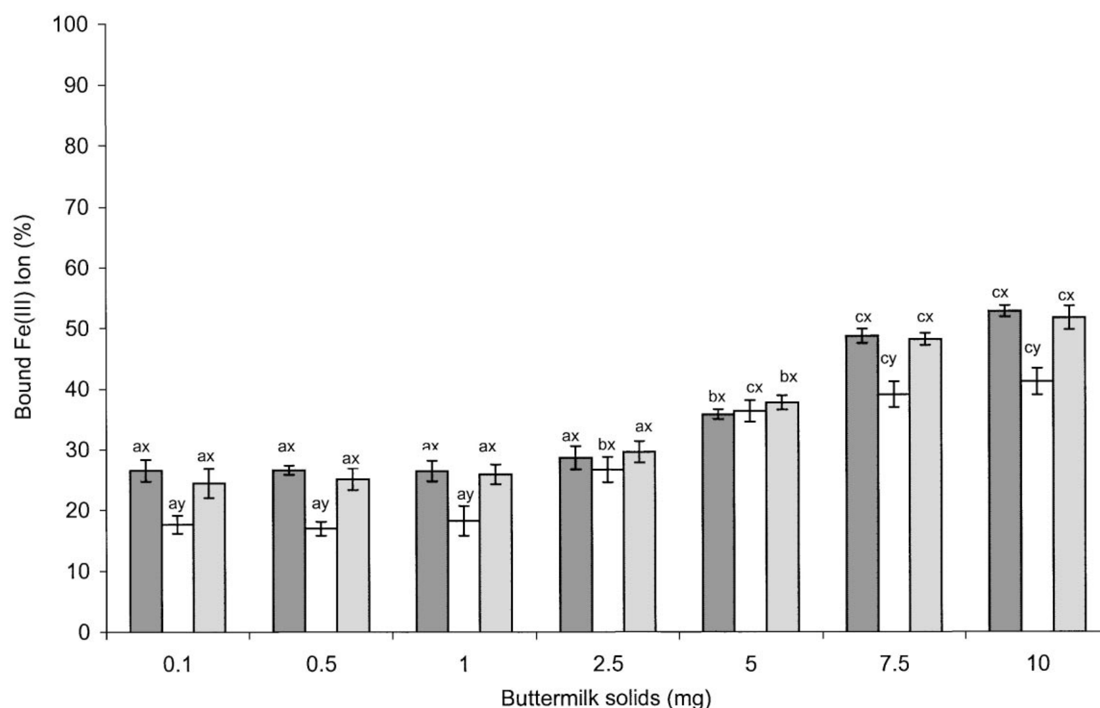

Fig. 9. Ferric iron binding by buttermilk solids (BMS). All values represents mean±SEM. [abc]Data within a treatment with different letter are significantly different ($P<0.05$). [xy]Data between treatments with different letter are significantly different ($P<0.05$). ■ 1.0 mM Fe^{2+}; □ 2.5 mM Fe^{2+}; □ 5.0 mM Fe^{3+}.

4. Conclusion

A modified ferric-thiocyanate method for measuring both Fe^{2+} and Fe^{3+} chelation by BMS and other standard food sequestering agents is reported. Reaction conditions for this modified assay require a minimum of 20 min incubation time, a solvent of 75% ethanol, or other solvents with equal polarity, and an optimum pH range of 4.5–5.5. Fe^{2+} and Fe^{3+} binding by phytic acid were both determined to be between 85 and 90% of the originally added iron. Significantly less iron in both valency states was bound by EDTA and especially citric acid. Both EDTA and citric acid demonstrated different affinities for the binding of Fe^{2+} and Fe^{3+}. Similar finding was also observed for BMS, where a higher binding affinity was exhibited towards Fe^{2+}. All levels of BMS tested were capable of binding between 60 and 90% of Fe^{2+} and a maximum of 55% of Fe^{3+}. BMS demonstrated exceptional ability in solubilizing iron by chelation.

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